

# Risk assessment of endophyte toxins

S.C. FINCH<sup>1</sup>, R. MUNDAY<sup>1</sup>, J.S. MUNDAY<sup>2</sup>, L.R. FLETCHER<sup>3</sup> and A.D. HAWKES<sup>1</sup>

<sup>1</sup>AgResearch Ltd, Ruakura Research Centre, PB 3123, Hamilton, New Zealand.

<sup>2</sup>Institute of Veterinary, Animal and Biomedical Sciences, Massey University, PB 11 222, Palmerston North, New Zealand.

<sup>3</sup>AgResearch Ltd, Lincoln Research Centre, PB 4749, Christchurch, New Zealand.

sarah.finch@agresearch.co.nz

## Abstract

Lolitrems B and epoxy-janthitrems were found in the fat of sheep grazing wild-type and AR37 endophyte-infected pastures, respectively. Epoxy-janthitrems were also detected in the milk of cows grazing AR37 pastures. In order to give preliminary information on the possibility that these endophyte toxins could pose a threat to human health, a 3-week toxicological trial has been conducted in mice. The results of this experiment suggest that, at the levels found in food, the endophyte toxins are unlikely to cause any safety concern.

**Keywords:** lolitrems, epoxy-janthitrems, endophyte, AR37, fat, milk, toxin residues, toxicology

## Introduction

Insect damage to pasture causes major losses in agricultural revenue in New Zealand. Productivity is increased, however, by use of grasses that are infected with certain endophytic fungi that produce chemicals that act as insect anti-feedants (Prestidge *et al.* 1982; Ball & Prestidge 1993). The most common such endophyte in New Zealand is *Neotyphodium lolii*, which is widely found in perennial ryegrass (*Lolium perenne* L.). Unfortunately, this fungus produces not only anti-feedants but also a family of mycotoxins called lolitrems (Fletcher *et al.* 1999). These are toxic to ruminant animals, causing a tremorgenic syndrome known as ryegrass staggers. More recently, a novel endophyte, AR37, has been inoculated into a range of ryegrass cultivars. AR37 offers excellent pest protection as well as good animal productivity and weight gain. This endophyte does not produce lolitrems, but produces a related group of toxins called epoxy-janthitrems (Tapper & Lane 2004). The latter substances are much less toxic than the lolitrems, and episodes of staggers recorded in animals grazing AR37 have been infrequent and of short duration (Fletcher 2005).

It has recently been shown that lolitrems B, the major tremorgenic mycotoxin produced by *Neotyphodium lolii*, is present in the fat of cattle fed a diet containing this toxin (Miyazaki *et al.* 2004). Uptake into fat is not unexpected, because of the highly lipophilic nature of lolitrems B. Epoxy-janthitrems are equally lipophilic, raising the possibility that these substances may be present in the fat of animals grazing AR37.

This report details finding lolitrems B and epoxy-janthitrems residues in the fat of sheep grazing wild-type and AR37 endophyte-infected pastures, respectively. Epoxy-janthitrems were also detected in the milk of cows grazing AR37 pastures. Estimates are made of the levels which could be ingested by the consumer. In order to provide preliminary information on the toxicology of lolitrems B and epoxy-janthitrems, a 3-week feeding study, based upon FDA Guidelines (U.S. Food and Drug Administration 2000), has been conducted in mice.

## Materials and Methods

### Sampling

Sheep were grazed on wild-type endophyte-infected pastures (10 animals, 42 days) or AR37-infected pastures (6 animals, 80

days). Animals were killed by captive bolt and samples of perirenal, mesenteric, subcutaneous and intramuscular fats taken.

Cows were grazed on AR37 pastures for 7 days before sampling. Milk was collected from 15 cows for 3 days (morning and afternoon milkings) and milk for each time period pooled and sampled.

### Sample preparation and analysis

**Fat analysis** Samples of frozen fat (1 g) were homogenised in dichloromethane (10 mL) for assay of lolitrems B, using a Polytron homogeniser. Because of the instability of the janthitrems in chlorinated solvents (Wilkins *et al.* 1992), fat samples were homogenised in ethanol for assay of these substances. Toxins were assayed by HPLC (Gallagher *et al.* 1985; Tapper & Lane 2004).

**Seed/diet analysis for epoxy-janthitrems** Samples of seed or AR37-containing mouse food were ground and aliquots (25 mg) extracted with acetone (1.5 mL) using a rotating wheel (1 hour). The extract was spun, and analysed by HPLC.

**Analysis of epoxy-janthitrems in milk** Milk was extracted with acetone (1:3) for 1 hour on a rotating wheel. The sample was then centrifuged and analysed by HPLC.

### Toxicology

**Animals** Swiss albino mice (20 male and 20 female, 3–4 weeks old) were used in the study. Animals were housed in groups of five in a temperature controlled room (21 ± 1°C) with a 12 hour light-dark cycle. They were allowed access to food and water *ad libitum*.

**Diets** Diets were based on Teklad Global 2016 mouse food (Harlan UK, Bicester, England), ground to a fine flour using an Udy cyclone sample mill. Pure lolitrems B (Miles *et al.* 1994) was added to give a concentration of 1.06 ppm. AR37 endophyte-infected seed (GA66, 73 ppm epoxy-janthitrems) was ground in the same way, and added to the ground mouse food at a concentration of 30%. The powders were mixed with water to form a paste, made into small cakes and dried in a fan oven at 40°C. Control diets (ground mouse food with no additions and ground mouse food to which 30% seed containing no endophyte (GN139, line N2115)) were prepared similarly. The diets were prepared twice-weekly during the course of the experiment.

**Feeding and experimental protocol** All animal manipulations were approved by the Ruakura Animal Ethics Committee established under the Animal Protection (code of ethical conduct) Regulations Act, 1987 (New Zealand). Mice were randomly divided into four groups each containing five female and five male mice. Group 1 – Control, group 2 – lolitrems B diet (1.06 ppm), group 3 – Nil endophyte (30%), group 4 – AR37 endophyte (30%). Body weights were recorded daily, and the mice were examined for any changes in appearance, movement or behaviour, and the food intake of each group of five mice was recorded. The mice were also tested for the presence of tremor

twice-weekly (Gallagher & Hawkes 1986) and the heart rate and blood pressure of each animal was measured weekly using a Visitech Systems BP-2000 blood pressure analysis system. Also on a weekly basis, motor control and balance was measured using a Columbus Rotamex 4/8 accelerating rotarod. At the end of the 3 week feeding period, mice were killed by CO<sub>2</sub> inhalation and blood samples taken by heart puncture using heparin as anticoagulant. Haematocrit values, haemoglobin levels, mean corpuscular volumes, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentrations, and total red and white cell counts were determined in whole blood, while plasma was analysed for activities of alanine aminotransferase (ALT),  $\gamma$ -glutamyltransferase (GGT) and for levels of total bilirubin, total protein, albumin, globulin and creatinine.

**Necropsy and histology** At necropsy, macroscopic changes were noted, and weights of brain, heart, kidneys, liver and spleen recorded, and expressed as a percentage of body weight. These tissues, together with the adrenal gland, lungs, pancreas, gastrocnemius, jejunum (3 mm section), ovary/uterus or testes, spinal cord (3 x 2mm sections), stomach (washed), thymus and urinary bladder were fixed, sectioned and stained for histological examination.

## Results

### Toxin levels in mouse diet and AR37 seed

HPLC analysis showed the AR37 endophyte-infected seed contained 73 ppm epoxy-janthitrems. HPLC analysis of the prepared diets showed both lolitrem B and epoxy-janthitrems to be present in a homogeneous manner throughout the mouse diet and that the toxins remained stable for at least 6 days following diet preparation.

### Endophyte toxin residues in fat

Lolitrem B and epoxy-janthitrems were detected in the fat of sheep grazing wild-type and AR37 endophyte-infected pastures, respectively. Of those animals grazing wild-type endophyte-infected pastures, eight out of ten were suffering staggers at the time of slaughter, with four being severely affected. Lolitrem B was detected in the fat of all of the animals with a range of 34-72 ppb, with the highest levels observed in the samples from the animals exhibiting severe staggers. Lolitrem B was found in all of the fat samples examined (intramuscular, peri-renal, mesenteric, subcutaneous), with little difference in concentration. Of the animals grazing AR37 endophyte-infected pastures, only one animal showed a mild tremor but epoxy-janthitrems were present in the fat of all the animals. The highest levels were found in peri-renal and intramuscular fat with smaller amounts in mesenteric and subcutaneous fats. The animal showing staggers had an epoxy-janthitrem level of 2330 ppb in peri-renal fat compared with levels of 542-949 ppb in the peri-renal fat of animals showing no staggers. Five epoxy-janthitrem derivatives were detected in fat, the same number and in similar proportions to that found in herbage.

### Epoxy-janthitrems in milk

In the six pooled milk samples that were analysed, epoxy-janthitrems were present at concentrations between 33 and 75 ng/mL.

### Toxicology trial

The appearance, movement and behaviour of the mice remained normal throughout the experimental period, and no tremors were detected at any time. There was no significant difference in food intake between mice fed diet with or without lolitrem B or

between mice fed diet containing nil endophyte seed or AR37 endophyte-infected seed. All mice gained weight during the experiment and there was no significant effect of the toxins on the bodyweight gain of either male or female mice.

Heart rate and blood pressure measurements showed no significant effect of the toxins. Similarly, no differences in rotarod performance were noted among the different treatment groups.

No significant differences in any of the haematological parameters were recorded, and no changes in plasma biochemistry indicative of a toxic effect were observed.

The relative weights of brain, heart, kidneys or spleen were not significantly different among the treatment groups, but the relative liver weights of mice fed lolitrem B were significantly lower than those of control animals ( $P < 0.03$  for males,  $P < 0.04$  for females). No effect on liver weights was seen in animals fed AR37 infected seed.

No lesions were recorded at necropsy, and no pathological changes were observed histologically.

## Discussion

This study has clearly shown that endophyte toxin residues are present in animals. Lolitrem B was detected in the fat of animals grazing wild-type endophyte-infected pastures and epoxy-janthitrems were detected in the fat of animals grazing AR37 endophyte-infected pastures. The levels of epoxy-janthitrems were much higher than those of lolitrem B. This difference is a reflection of the higher concentrations of epoxy-janthitrems present in herbage. A wild-type endophyte-infected pasture is considered to be toxic when lolitrem B levels reach 2.5 ppm but in contrast levels of up to 50 ppm epoxy-janthitrems have been found in AR37 pastures (Finch, S. pers. comm.). The relatively low incidence of tremors in animals grazing AR37 pastures despite the high toxin concentration is consistent with the epoxy-janthitrems having a low tremorigenic potential. Epoxy-janthitrem residues were also detected in milk.

The presence of toxin residues in food requires an assessment as to whether these could pose a problem to human health. In the 3-week toxicological study in mice, no adverse effects were recorded with either lolitrem B or the epoxy-janthitrems. The relative liver weights of both male and female mice fed lolitrem B were lower than those of control animals. Liver enlargement is a common occurrence in rodent toxicity studies, often indicating enzyme induction required for xenobiotic metabolism (Amacher *et al.* 1998), but the significance of the observed decrease in liver weight in the test animals is presently unknown. The effect, although statistically significant, was small, however, and in the absence of any indication of liver damage, it is considered of no toxicological significance.

The daily dose rates of the toxins consumed by the mice were calculated from daily food consumption figures and body weights, averaged over the 3 week experimental period. Mice consumed 0.165 mg/kg/day lolitrem B or 3.9 mg/kg/day epoxy-janthitrems. To determine the relevance of these dose levels to the human situation, it is necessary to estimate how much toxin a human could eat. If a person ate 500 g of meat containing 20% fat per day and drank 1 litre of milk per day, which contained the highest levels of toxin found in our experiment (75 ppb lolitrem B in fat, 2330 ppb epoxy-janthitrems in fat and 75 ng/mL epoxy-janthitrems in milk), that person would consume 7.5  $\mu$ g of lolitrem B or 308  $\mu$ g of epoxy-janthitrems per day. Assuming a body weight of 50 kg, the intake of lolitrem B would be 0.00015 mg/kg/day and that of epoxy-janthitrems 0.00616 mg/kg/day. The doses administered to the mice were respectively 1100 times

and 633 times greater than these possible human intakes, which are based on a worst-case scenario where a light person consumes a very fatty diet. These results suggest that, at the levels found in food, the endophyte toxins are unlikely to pose any safety concern. In order to provide further information on this point, an additional mouse study, in which the toxins will be administered over a 90-day period, is planned.

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